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Prospecting for new group A streptococcal vaccine candidates

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***Background & objectives*: Most group A streptococcal (GAS) vaccine strategies focused on the surface M protein of the GAS. However, vaccine based on M protein have some drawbacks. In the present study, we used two approaches to identify new proteins and peptides that may have utility as vaccine candidates.**

***Methods*: A whole gel elution procedure was used to separate GAS surface antigens into 9 size fractionated pools. Mice were vaccinated with each pool and antibody titre, opsonic ability and protective capacity measured. In an alternative approach BioInformatics was used to identify putative GAS surface proteins. Peptides from within these proteins were then selected on the basis of predicted antigenicity or location. These peptides were conjugated to keyhole lymphocyanin (KLH) and immunogenicity measured in a mouse model.**

***Results*: One pool of GAS surface proteins (approximately 29kDa) induced antibodies that were both opsonic and potentially protective. Immunoflourescent microscopy demonstrated that these antibodies bound to the surface of M1 GAS. Amino acid sequencing subsequently identified superoxide dismutase as the major antigen in this pool. A BioInformatic search of the M1 GAS genome and subsequent analysis identified several peptides that fulfilled criteria as potential vaccine candidates. Each peptide when conjugated to KLH was able to induce a strong antibody response.**

***Interpretation & conclusion*: Several new antigens were identified that may have potential as vaccine targets. A future GAS vaccine may have multiple peptide epitopes, providing protection against multiple GAS strains.**

Key words GAS - potential vaccine - superoxide dismutase - surface proteins

Streptococcus pyogenes or group A streptococcus (GAS) is a human bacterial pathogen that colonises the throat or skin surfaces of the host. GAS infection may result in a number of diseases including pharyngitis (classical 'strep throat'), impetigo and less commonly necrotising fasciitis among others. If treated ineffectively, the post-streptococcal sequelae acute rheumatic fever (ARF) and acute glomerulonephritis may develop. ARF in its severest form leads to the potentially fatal rheumatic heart disease (RHD). While the incidence of rheumatic fever and serious GAS infection remains low in developed countries, there has been an increase in the number of cases reported since the mid 1980s[1]. Of greater concern is the prevalence of GAS infection and ARF in the developing countries. Rheumatic fever is a leading cause of heart disease in children in these regions[2,3].

Most group A streptococcal vaccine strategies have focused on the surface M protein, a major virulence factor of GAS[4,5]. Antibodies to the amino terminus of the M protein have been shown to be opsonic and provide protection against challenge from homologous organisms; *i.e.*, they are type specific. However, vaccines based on

the M protein face two major obstacles. Firstly, the amino terminus of the M protein is highly antigenically variable. There are greater than 100 reference GAS *emm* types and an increasing number of which are not M serotypable. Antibodies to the serotypic specific amino termini are therefore ineffective in providing broad spectrum protection against multiple GAS strains. Secondly, some epitopes from the M protein are known to be immunologically cross-reactive with heart proteins and possibly contribute towards the pathology of RHD[3,6]. Current strategies in utilising the M protein as a vaccine involve the use of multiple N-termini and/or defined epitopes within the conserved carboxyl C-region that are not cross-reactive with host tissues[7,8]. Apart from the M protein only a few antigens are under development as potential GAS vaccine candidates. These include GAS carbohydrate, C5a peptidase and cysteine protease[9-11].

We hypothesised that GAS vaccine antigens should be surface exposed. They should also be immunogenic, and ideally induce opsonic antibody responses. In the present study two approaches were used for the identification of non M protein streptococcal antigens that may have potential as vaccine candidates. The first involved the screening of pools of GAS surface proteins for antigens that invoked antibodies with properties desirable in a GAS vaccine. The second involved the use of BioInformatics to predict antigenic peptides encoded by open reading frames found in the M1 GAS genome.

Material & Methods

Preparation of cell surface extracts: M1 GAS was grown overnight in Todd Hewitt broth. Extracts of M1 GAS surface proteins were prepared using mutanolysin digestion as described[12,13]. Surface protein extracts were then separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and isolated into individual fractions using a whole gel eluter (Bio-Rad, USA).

Selection of peptides: Many surface proteins of Gram-positive bacteria have a characteristic 'LPxTG' amino acid motif in the C-terminal region. Surface proteins additionally generally have an export signal sequence that can be used to predict whether a protein is transported to the bacterial cell surface[14]. The M1 GAS genome has 1,752 putative protein encoding regions[15]. Using a BioInformatical approach all 1,752 open reading frames were analysed for the presence of an 'LPxTG' motif (or close homologue) and export signal motif. A number of proteins were identified with one or both of these motifs, termed 'putative surface proteins (PSPs). It was hypothesised that the amino terminus would be the most immunologically exposed portion of the molecule. Consequently, the amino terminus of the predicted mature PSP (*i.e.*, excluding putative signal sequence) was analysed for the presence of antigenic amino acid sequences as predicted by computational algorithms and peptides from these regions were synthesised. Where algorithms failed to identify sequences of high antigenicity, peptides corresponding to the extreme amino terminus (*ex* signal sequence) of the PSP were synthesised. The peptides used in this study are listed in Table I.

Immunological protocols: Outbred Quackenbush mice (n=5) were subcutaneously immunized with antigen following established protocols. In the case of peptides, antigens were first conjugated to keyhole lymphocyanin

Table I. Peptides used in this study

Peptide	Putative protein function	Genbank accession no.
ACHNTSKPSNTDSVFSLTGK	unknown	AAK33302.1
AQNFRNIMHGSDSFFYTFTS	putative ABC transporter, permease protein	AAK34838.1
CSTLVEKDVAPKDELEMLAW	putative membrane protein	AAK34293.1
ESVLQAQMAAQQLPVIGGIA	putative sortase	AAK34025.1
LEALADQTDALQSEEAAVVK	protein GRAB	AAK34185.1
QEVFSLVKEPILKQTQASSS	Unknown	AAK33772.1

(KLH). The primary immunization consisted of antigen emulsified in phosphate buffered saline (PBS). Boosts were delivered in PBS. Qualitative ELISA and opsonisation assays were performed as previously described[16]. Immunofluorescent assays were carried out essentially as described[7] with an additional step in which cells were preincubated with human immunoglobulin to inhibit the non-specific binding of antigen-specific antisera by Ig binding proteins and Fc-binding receptors on the surface of GAS[17].

Results

Identification of immunogenic antigens in pooled protein fractions: M1 GAS surface protein extract prepared by mutanolysin digestion were size fractionated by SDS-PAGE whole gel elution. Neighbouring fractions containing proteins of similar size were then pooled to give a total of 9 groups (defined as Pool A to Pool I) with each pool containing several proteins. The molecular weight of the protein in these pools ranged from approximately 20 to 78 kDa. Each of the pools was used to subcutaneously immunize mice and antibody response measured via an indirect ELISA against the whole M1 GAS surface protein extract (Table II). All Pools invoked a strong antibody response, with antibody titres from mice within some pools exceeding the range of the experiment (titres $>3.3x10^6$). At the highest dilution used in the ELISA, optical density was greatest against pool B and pool D antisera.

All antibodies were subsequently tested for their ability to bind to the surface of GAS, to opsonise GAS and to protect vaccinated mice against subsequent GAS challenge (Table II). Immunoflourescent microscopy showed that antisera raised against each of the pools bound to the surface of GAS (as compared to normal mouse sera). Antisera raised against pool G elicited the most intense fluorescence, with pools E and F also showing a high degree of fluorescence. Pools F and G antisera showed the highest level of opsonic ability (58 and 62% respectively) with the difference between these two pools not statistically significant. To investigate the level of protection conveyed by antibodies raised to the pooled fractions, mice were challenged with 400µl of passaged M1 GAS ($3x10^3$ cfu/ml), and monitored over a 10-day period. After 10 days, 40 per cent of the PBS immunized mice and 80 per cent of mice immunized with a positive control antigen were protected from challenge. Mice immunized with pool G exhibited the greatest level of protection from M1 GAS challenge, with 100 per cent survival. Pools D, E, and F also showed high levels of protection (>75%). Due to the low numbers of mice within each group, differences in murine survival between groups were found not to be statistically significant.

The data suggests that pool G elicits contain a surface antigen that showed antibody responses which were both opsonic and potentially protective. The size of the major antigen in this pool was approximately 29 kDa. Amino acid sequencing identified this antigen as superoxide dismutase (SOD). This antigen is a known virulence factor of many bacteria[18].

Immunoreactivity of peptide conjugates: ELISA was used to measures peptide specific antibody responses for each of the peptides used in this study (Fig.). The mean titre for LEA20-KLH and QEV20-KLH when measured against there respective peptides was greater than $2x10^6$. In contrast titres to AQN20-KLH was only 4300 ± 1700. As some peptides are known not to adhere to microtitre plates, AQN20 antibody titres were next measured against AQN20 linked to diphtheria toxoid (DT). The titres of AQN20 against AQN2-DT was

Table II. Properties of surface fractions and induced antibodies

	Pool								
	A	B	C	D	E	F	G	H	I
Molecular weight (kDa)	78	60	55	40	35	31	29	23	20
Opsonic activity (%)	42	41	39	45	36	58	62	17	6
Immunofluorescence*	+	+	+	++	++	++	+++	+	+
Challenge (% survival)	40	40	60	75	80	80	100	60	40

*Relative fluorescence; +, low fluorescence; ++, medium fluorescence; +++, high fluorescence

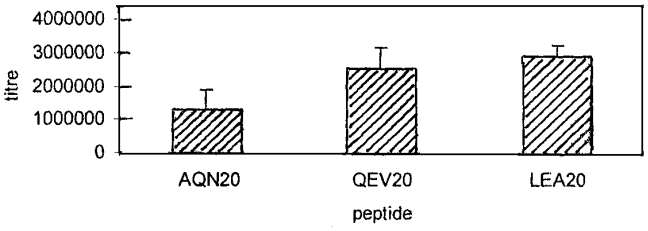

Fig. Average peptide specific antibody titres in mice.

found to be greater than $1.3x10^6$. The average titre in this group against DT was only 300.

Discussion

We used two approaches for the identification of antigens that may be of use as potential GAS vaccine candidates. Screening of pools of proteins purified from the surface of GAS identified SOD as a potential vaccine candidate. This approach had the benefit of directly testing many antigens for properties that are considered desirable in a vaccine (*e.g.*, opsonic ability). A potential drawback was that each pool contained a number of antigens, each of which might partially contribute to protection. Additionally proteins present in low abundance in the surface extracts, or not expressed in the bacterial growth conditions used may be overlooked in this system. Nevertheless this approach identified SOD as a potential GAS vaccine candidate. This enzyme is a major virulence determinant in other bacteria[18-20], and has also been shown to induce protective immune responses[21-23]. SOD is also highly conserved across streptococci[24] and inactivation of this gene has been shown to severely limit the growth capacity of GAS[25]. We are currently assessing the utility of recombinantly expressed SOD to induce antibody responses which are opsonic and protective.

The use of purified antigens overcomes many of the limitations of the above method. The use of peptides instead of whole protein also minimises the amino acid sequence in a vaccine candidate, reducing the chance of engendering host cross-reactive antibodies. We hypothesised that peptides derived from proteins located on the surface of GAS are exposed to the host immune system. Using BioInformatics six peptides were identified with this profile and found to be immunogenic in mice. We are currently testing these antibodies in opsonisation experiment against several GAS strains. A future GAS vaccine may contain multiple peptide epitopes, providing protection against broad multiple GAS strains.

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